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PATENT APPLICATION

ENHANCED PHOSPHOLIPID REDUCTION AND CALCIFICATION MITIGATION OF BIOLOGICAL MATERIALS

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CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to commonly assigned U.S. provisional patent application number 60/396,879 filed July 16, 2002, which is incorporated herein by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Implantation of medical devices into humans and other animals is carried out with increasing frequency. Implantable medical devices may be formed wholly or partially of biological tissue that has been chemically "fixed" or preserved. The technique used for chemical fixation of biological tissues typically requires exposure of the biological tissue to one or more chemical agents that are capable of forming cross-linkages between connective tissue protein molecules present in the tissue. Examples of fixed biological tissues that have been used to form implantable bioprostheses include cardiac valves, blood vessels, skin, dura mater, pericardium, ligaments and tendons.

[0003] The heart valve is one of the most widely studied implantable prosthesis and its clinical application and pathology are well documented (Schoen et al., *Cardiovascular Pathology* **1**:29-52 (1992)). More than 100,000 cardiac valve prostheses are placed in patients each year (Levy, et al., U.S. Patent Number 5,674,29). The most common bioprosthetic heart valves in current use around the world are made of porcine aortic tissue or bovine pericardium tissue (Seifter, et al., U.S. Patent Number 5,476,516).

[0004] Biological tissues, including porcine aortic and bovine pericardium tissue, typically contain connective tissue proteins (i.e., collagen and elastin), which act as the supportive framework of the tissue. The pliability or rigidity of each biological tissue is largely determined by the relative amounts of collagen and elastin present within the tissue and/or by the physical structure and conformation of its connective tissue frame work. Each collagen molecule is made up of three polypeptide chains intertwined in a coiled helical conformation.

[0005] To stabilize the bioprosthetic tissue, the reactive moieties of the tissue are blocked by process known as fixation. In 1968, it was found that collagen was stabilized by aldehydes (Nimni et al., *J. Biol. Chem.* **243**:1457-1466 (1968)). Aldehydes stabilize collagen

by forming chemical cross-linkages between the polypeptide chains within a given collagen molecule (i.e., intramolecular crosslinkages), or between adjacent collagen molecules (i.e., intermolecular crosslinkages). Today, various chemical fixative agents, sometimes referred to as tanning agents are known in the art. Fixative agents that have been utilized to cross-link collagenous biological tissues include aldehydes (e.g., formaldehyde, glutaraldehyde, dialdehyde starch, para formaldehyde, glyceraldehyde, glyoxal acetaldehyde, acrolein), diisocyanates (e.g., hexamethylene diisocyanate), carbodiimides, and certain polyepoxy compounds (e.g., Denacol-810, -512, or related compounds). Photooxidation has also been used to fix biological tissue.

[0006] Of the various chemical fixatives available, glutaraldehyde is used as the fixative for many commercially available bioprosthetic products, including porcine bioprosthetic heart valves (i.e., the Carpentier-Edwards™ stented porcine bioprosthesis; Edwards Lifesciences, Irvine, Calif. 92614), bovine pericardial heart valve prostheses (e.g., Carpentier-Edwards™ Perimount Pericardial Bioprosthesis, Edwards Lifesciences; Irvine, Calif. 92614) and stentless porcine aortic prostheses (e.g., Edwards™ PRIMA Plus Stentless Aortic Bioprosthesis, Edwards Lifesciences, Irvine, Calif. 92614).

[0007] A primary failure mode of bioprosthetic tissue implantation, including both porcine and pericardial biological replacement heart valves, is tissue calcification. For example, calcification causes more than 50 percent of heart valve bioprostheses failures within 10 years of implantation (Schoen et al., *Cardiovascular Pathology* **1**:29-52 (1992)). In addition, calcification proceeds more rapidly in children than in adults (*Id.*). Morphological examination of explanted bioprostheses indicates that calcification is frequently associated with collagen fibrils, organelles such as mitochondria and nuclei of devitalized cells (including connective tissue cells of the leaflets, and cells of the muscle shelf, and aortic wall of porcine aortic valves), thrombi, and vegetations (Schoen FJ, et al., *J Card Surg* **9**[suppl]:222-227 (1994); Schoen FJ, et al., *J. Biomed. Mater. Res.* **47**:439-465 (1999); Ferrans YJ, et al., *Hum Pathol* **18**:586-595 (1987)). Although the calcification process itself has been studied extensively, the mechanism of tissue calcification remains unclear.

[0008] Calcification can result in undesirable stiffening or degradation of the bioprosthesis. Two types of calcification-intrinsic and extrinsic--are known to occur in fixed collagenous bioprostheses. Intrinsic calcification is characterized by the precipitation of calcium and phosphate ions within the fixed bioprosthetic tissue, including the collagen matrix and

remnant cells. Extrinsic calcification is characterized by the precipitation of calcium and phosphate ions within the thrombus, including adherent cells (e.g., platelets) that adhere to the bioprosthesis and the development of calcium phosphate-containing surface plaques on the bioprosthesis.

[0009] The factors that affect the rate at which fixed tissue bioprostheses undergo calcification have not been fully elucidated. However, factors that are thought to influence the rate of calcification include:

a) patient's age;

b) existing metabolic disorders (i.e.,

c) hypercalcemia, diabetes, etc.);

d) dietary factors;

e) infection;

f) parenteral calcium administration;

g) dehydration;

h) distortion/mechanical factors;

i) inadequate coagulation therapy during initial period following surgical implantation; and

j) host tissue responses.

[0010] The factors that are thought to affect the propensity for platelets to adhere to a fixed bioprosthetic tissue include:

a) tissue damage;

b) diet;

c) surface properties of the tissue, including the nature of exposed collagen (e.g., type I, IV, etc.);

d) metabolic changes;

e) coagulation;

f) hemodynamics

g) inflammation; and,

h) infection.

[0011] Various techniques have heretofore been proposed for reducing the *in situ* calcification of glutaraldehyde-fixed bioprostheses. Included among these calcification reduction techniques are the methods described in Nashef et al., U.S. Patent Number

4,885,005; Carpentier et al., U.S. Patent Number 4,648,881; Girardot et al., U.S. Patent Number 4,976,733; Carpentier et al., U.S. Patent Number 5,002,2566; Pollock et al., EP 103947A2; Nashef et al., U.S. Patent Number 4,885,005, Cunanan et al., U.S. Patent Number 6,214,054, and Cunanan et al., U.S. Patent Application Number 09/693,186, which are herein incorporated by reference in their entirety for all purposes.

[0012] However, there continues to be a need in the art for methods for producing a bioprosthesis resistant to undesirable stiffening or degradation. The present invention meets these and other needs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 provides data from experiments to assess 90-day rat subcutaneous pericardial leaflet calcification (μg Ca/mg dry wt) as described in Example 1 below.

[0014] Figure 2 illustrates data from experiments to assess 90-day rat subcutaneous pericardial leaflet calcification (μg Ca/mg dry wt) as described in Example 1 below.

[0015] Figure 3 provides data from experiments to assess rat 90-day subcutaneous porcine leaflet calcification (μg Ca/mg dry wt) as described in Example 2 below.

[0016] Figure 4 illustrates data from experiments to assess 90-day rat subcutaneous porcine leaflet calcification (μg Ca/mg dry wt) as described in Example 2 below.

BRIEF SUMMARY OF THE INVENTION

[0017] The present invention provides methods of producing superior biological materials for implantation by mitigating calcification, reducing calcification, and/or reducing phospholipid content in the biological materials upon implantation. The procedures provide biological tissues that are well suited for use in bioprosthetic devices. The invention further provides superior biological materials prepared in accordance with the methods of the invention, and implantable prosthetics that comprise such superior biological materials.

[0018] Thus, in a first aspect, the present invention provides a method of treating a biological material, which comprises contacting the biological material with a preparation that contains a cross linking agent and a surfactant. The preparation does not contain a denaturant.

[0019] In a second aspect, the present invention provides a method of treating a biological material, which comprises the step of contacting the biological material with a preparation

having a surfactant and a cross linking agent in the absence of a denaturant (hereinafter referred to as the SCL step); and the step of contacting the biological material with a preparation comprising a surfactant, a cross linking agent and a denaturant (hereinafter referred to as the SCLD step).

[0020] In a third aspect, the present invention provides a method of treating a biological material that comprises four separate steps performed in a specified sequence. In step one, the biological material is contacted with a preparation containing a cross linking agent in the absence of denaturant and surfactant. After step one, step two is performed, which comprises contacting the biological material with a preparation containing a cross linking agent and a surfactant in the absence of denaturant. After step two, step three is performed, which comprises contacting the biological material with a preparation containing a cross linking agent, a denaturant and a surfactant. After step three, the biological material is contacted with a terminal liquid sterilization solution.

[0021] In a fourth aspect, the present invention provides a method of treating a biological material that comprises contacting the biological material with a preparation having a cross linking agent and a surfactant in the absence of a denaturant. The method results in mitigation of calcification of the biological material upon implantation into a host organism relative to an identical method of treating the biological material with the exception that the biological material is not contacted with a preparation having a cross linking agent and a surfactant in the absence of a denaturant.

[0022] In a fifth aspect, the present invention provides a method of treating a biological material that comprises contacting the biological material with a preparation having a cross linking agent and a surfactant in the absence of a denaturant. The method results in reduction of phospholipid content of the biological material upon implantation into a host organism relative to an identical method of treating the biological material with the exception that the biological material is not contacted with a preparation having a cross linking agent and a surfactant in the absence of a denaturant.

[0023] In a sixth aspect, the present invention provides a method of treating a biological material that comprises contacting the biological material with a preparation having a cross linking agent and a surfactant in the absence of a denaturant. The method results in reduction of calcium content of the biological material upon implantation into a host organism relative to an identical method of treating the biological material with the exception that the

biological material is not contacted with a preparation having a cross linking agent and a surfactant in the absence of a denaturant.

[0024] In a seventh embodiment, the present invention provides a biological material resistant to calcification produced by a method of treating the biological material comprising the step of contacting the biological material with a preparation comprising a surfactant and a cross linking agent in the absence of a denaturant; and the step of contacting the biological material with a preparation comprising a surfactant, a cross linking agent and a denaturant.

[0025] These and other embodiments and advantages of the present invention will be apparent from the detailed description that follows.

DETAILED DESCRIPTION OF THE INVENTION

METHODS OF MITIGATING OR REDUCING CALCIFICATION

[0026] In one aspect, the present invention provides superior methods of treating a biological material, wherein the treatment mitigates calcification when the biological material is implanted into a host organism. The methods of treatment to mitigate calcification are also referred to herein as methods of mitigating calcification of a biological tissue. The methods of the present invention mitigate calcification of a biological material relative to methods that are identical with the exception that the SCL step is not performed. Individual steps of the methods of the present invention are described in detail below.

[0027] Mitigation of calcification refers to reducing the incidence of calcification. The incidence of calcification refers to the number of members of a population of implanted biological materials for which calcification occurs relative to the total number of members of the population. Elimination of calcification means mitigating calcification wherein no members of a population of implanted biological materials are calcified.

[0028] An exemplary calcification is the accumulation of calcium sufficient to cause stiffening or degradation of biological tissue implanted into a host organism. A variety of techniques are useful in measuring calcification in a biological material after implantation. In an exemplary assay, calcification is measured by implanting the biological material in a host organism for a period of time, removing the implanted biological material, and measuring the calcium levels in the biological material. The biological material may be implanted subcutaneously or intramuscularly in, for example, a mammalian host organism between 10 and 100 days. The mammalian host organism may be any appropriate species, such as a rat or a rabbit.

[0029] Another exemplary calcification is the measurement of calcium accumulation after implantation of the biological material into a mammalian host organism for 30 to 90 days. The biological material is then removed and analyzed for calcium content. Surprisingly, in this example, biological material containing calcium levels below 5 μg of calcium per mg of dried material (hereinafter referred to as μg Ca/mg) have superior mechanical properties, enhanced longevity and durability, and increased resistance to failure when used as bioprosthetic tissue compared to biological materials containing more than 5 μg Ca/mg. Conversely, biological material containing calcium levels above 5 μg Ca/mg have inferior mechanical properties, decreased durability, and increased rates of failure when used as bioprosthetic tissue. Therefore, in this example, bioprosthetic or biological tissue containing calcium content above 5 μg Ca/mg tissue is considered calcified whereas bioprosthetic or biological tissue containing calcium content below 5 μg Ca/mg tissue is considered not calcified. Thus, in this example, mitigation of calcification is defined as the reduction in the incidence of a biological material having greater than 5 μg Ca/mg dried material when implanted into a mammalian host organism for about 30 to about 90 days.

[0030] In another exemplary embodiment, calcification is measured by subcutaneously implanting the biological material into a rat for about 90 days. The bioprosthetic or biological tissue containing calcium above 5 μg Ca/mg tissue is considered calcified whereas bioprosthetic or biological tissue containing calcium below 5 μg Ca/mg tissue is considered not calcified. Thus, in this example, mitigation of calcification is defined as the reduction in the incidence of a biological material having greater than 5 μg Ca/mg material when implanted subcutaneously into a rat for about 90 days.

[0031] In another exemplary embodiment, mitigation of calcification results in elimination of calcification. The term "elimination of calcification" or "eliminating calcification" as used herein, means mitigating calcification wherein no members of a population of implanted biological materials are calcified.

[0032] In another aspect, the present invention provides methods of treating a biological material, wherein the treatment reduces calcium content when the biological material is implanted into a host organism. The methods of treatment to reduce calcium content are also referred to herein as methods of reducing calcium content of a biological tissue. The methods of the present invention reduce calcium content of a biological material relative to methods

that are identical with the exception that the SCL step is not performed. Individual steps of the methods of the present invention are described in detail below.

METHODS OF REDUCING PHOSPHOLIPID CONTENT

[0033] In another aspect, the present invention provides methods of treating a biological material, wherein the treatment reduces phospholipid content of the biological material. The methods of treatment to reduce phospholipid content are also referred to herein as methods of reducing phospholipid content of a biological tissue. The methods of the present invention reduce phospholipid content of a biological material relative to methods that are identical with the exception that the SCL step is not performed. Individual steps of the methods of the present invention are described in detail below.

[0034] Various methods of reducing phospholipid content in a biological material are useful in the current invention. For example, phospholipid content of a biological material may be measured by extracting the tissue with a solvent mixture to solubilize the phospholipids. Exemplary phospholipids measured include phospholipids present in the tissue due to the cellular nature of the tissue.

[0035] Phospholipid content of a biological material may be correlated to desired or undesired properties resulting from implantation of the biological material. For example, phospholipid content of a biological material may be measured and the biological material may then be implanted into a host organism. The properties of the biological material may then be analyzed. In an exemplary embodiment, phospholipid content is measured and the biological material is subsequently implanted into a mammalian host organism for 30 to 90 days. Surprisingly, in this example, biological material containing phospholipid content below 1 μg of phospholipids per mg of dried material (hereinafter referred to as μg PL/mg) have superior mechanical properties, enhanced longevity and durability, and increased resistance to failure when used as bioprosthetic tissue compared to biological materials containing more than 1 μg PL/mg. Conversely, biological material containing phospholipid content above 1 μg PL/mg have inferior mechanical properties, decreased durability, and increased rates of failure when used as bioprosthetic tissue.

[0036] In another exemplary embodiment, phospholipid content is measured and the biological material is subsequently implanted into a rat for about 90 days. In this example, desirable biological material contained less than 1 μg PL/mg and preferably less.

[0037] In another exemplary embodiment, biological material containing less than 0.4 μg PL/mg exhibits desirable properties after implantation into a rat for about 90 days.

[0038] In an exemplary assay, phospholipid content is measured by quantifying selected phospholipids within a sample. A variety of phospholipids may be selected for quantification, including acidic phospholipids, basic phospholipids and neutral phospholipids. In another exemplary embodiment, the selected phospholipids are sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid. In another exemplary embodiment, the selected phospholipids include gangliotetraosylceramide (Gg4).

[0039] Thus, in another aspect, the present invention provides methods of treating a biological tissue, wherein the treatment reduces phospholipid content of the biological tissue when implanted into a host organism. The methods of the present invention reduce phospholipids of a biological material relative to methods that are identical with the exception that the SCL step is not performed. Individual steps of the methods of the present invention are described in detail below.

METHODS OF THE PRESENT INVENTION

[0040] The methods of the present invention provide superior methods for mitigating calcification, reducing calcification, and/or reducing phospholipid levels in an implanted biological material. The methods of the present invention include the step of contacting the biological material with a preparation comprising a surfactant and a cross linking agent without denaturant (hereinafter referred to as the SCL step). In a further embodiment, the methods of the present invention include the step of contacting the biological material with a preparation comprising a surfactant, a cross linking agent and a denaturant (hereinafter referred to as the SCLD step).

[0041] In an exemplary embodiment, the methods of the present invention further include, after completion of the SCL and SCLD steps previously discussed, contacting the biological tissue with a terminal liquid sterilization solution.

[0042] In another exemplary embodiment, the methods of the present invention further include, before performing the SCL and SCLD steps, contacting the biological tissue with a fixation solution that comprises a cross linking agent in the absence of denaturant.

[0043] In another aspect, the present invention provides a method of treating a biological material including the steps of:

[0044] (a) first contacting the biological material with a preparation comprising a cross linking agent;

[0045] (b) following (a), contacting the biological material with a preparation comprising a surfactant and a cross linking agent (the SCL step);

[0046] (c) following (b), contacting the biological material with a preparation comprising surfactant, a cross linking agent and a denaturant (the SCLD step); and

[0047] (d) following (c), contacting the biological material with a terminal liquid sterilization solution.

[0048] Exemplary embodiments of these and other steps are discussed in detail below.

Surfactants

[0049] Methods of the present invention include contacting a biological material with a surfactant, either alone or in combination with other chemical agents. The treatment can be performed as a single step. Alternatively, the treatment can be performed as a series of sequential steps. At the end of each sequential step, the surfactant is preferably removed and a new fraction of surfactant is added. Surfactants of use in the present invention include any surfactant, either substantially pure or containing additives. An exemplary surfactant is a surface active agent capable of reducing the surface tension of water. Exemplary defining features of surfactants can be found in KIRK-OTHMER'S "ENCYCLOPEDIA OF CHEMICAL TECHNOLOGY," Third Edition, Vol. 22, pp. 332-432, which is hereby incorporated by reference.

[0050] Other exemplary surfactants include anionic, cationic, nonionic, and amphoteric surfactants. In an exemplary embodiment, the method of the present invention uses a nonionic surfactant as the surfactant. Nonionic surfactants include, without limitation, various ethoxylates, carboxylic acid esters, glycol esters, polyoxyethylene esters, anhydrosorbitol esters, ethoxylated anhydrodrosorbitol esters, glycerol esters of fatty acids, carboxylic amides, diethanolamine condensates, and the like. Exemplary nonionic surfactants also include ethoxylated natural fats, oils and waxes. The skilled practitioner will recognize that many other surfactants are suitable for use in the method of the present

invention. A list of additional surfactants can be found in U.S. Patent No. 6,214,054, which is incorporated by reference in its entirety for all purposes.

[0051] The ethoxylated natural fats, oils, and waxes are optionally from a group including, without limitation, lauric, oleic, stearic, and palmitic fatty acids having trade names such as Armotan, Emsorb, Glycosperse, Hodag, and Tween.

[0052] In another exemplary embodiment, the surfactant is polyoxyethylene sorbitan monooleate (Tween 80). Surfactants such as Tween 80 are widely used in biochemical applications including: solubilizing proteins, isolating nuclei from cells in culture, growing of tubercule bacilli, and emulsifying and dispersing substances in medicinal and food products.

[0053] In an exemplary embodiment, the tissue is treated with a preparation or solution containing from about 0.1% to about 50% surfactant by weight. In another exemplary embodiment, the tissue is treated with a preparation or solution containing from about 0.1% to about 10% surfactant. In another exemplary embodiment, the tissue is treated with a preparation or solution containing more than 0.6% and less than 50% surfactant. In another exemplary embodiment, the preparation or solution contains from about 1.7% to about 25 % surfactant. In another exemplary embodiment, the preparation or solution contains 0.7%, 0.8%, 0.9%, 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, or 2.9% surfactant. In another exemplary embodiment, the preparation or solution contains 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% surfactant.

Cross Linking Agents

[0054] Methods of the present invention include contacting a biological material with a cross linking agent, either alone or in combination with other chemical agents. The treatment can be performed as a single step. Alternatively, the treatment can be performed as a series of sequential steps. At the end of each sequential step, the cross linking agent is preferably removed and a new fraction of cross linking agent is added. Exemplary cross linking agents include compounds capable of stabilizing the structure of a biological material through the formation of covalent bonds.

[0055] Exemplary cross-linking reagents include, but are not limited to, aldehydes (e.g., formaldehyde, glutaraldehyde, dialdehyde starch, para formaldehyde, glyceraldehyde, glyoxal acetaldehyde, acrolein), diisocyanates (e.g., hexamethylene diisocyanate), carbodiimides (e.g. dicyclohexylcarbodiimide, 1-ethyl-3-[3-dimethylaminopropyl]

carbodiimide hydrochloride), polyepoxy compounds (e.g., Denacol-810, -512), bifunctional maleimide compounds (e.g. 1,4-Bis-maleimidtyl-2,3-dihydroxybutane, N-(β-maleimidopropyloxy) sucinimide ester), bifunctional N-hydroxysuccinimide ester compounds (e.g. disuccinimidyl glutarate, 3,3'-dithiobis(sulfosuccinimidyl propionate)), bifunctional imidoester compounds (e.g. dimethyl suberimidate), bifunctional pyridylthio compounds (e.g. 1,4-di-[3'-(2'-pyridylthio)-propionamido]butane), bifunctional vinylsulfone compounds (e.g. 1,6-hexane-bis-vinylsulfone) and photoactivatable cross-linkers (e.g. azides, phenyl azides).

[0056] In an exemplary embodiment, the cross linking agent is an aldehyde, including both mono- and poly-aldehydes. Aldehydes of use in practicing the present invention include any aldehyde, either substantially pure or containing additives. Exemplary aldehydes include one or more compounds of the following: acetaldehyde, butyraldehyde, isobutyraldehyde, propionaldehyde, α-methylpropionaldehyde, 2-methylbutyraldehyde, cyclopentanecarbaldehyde, benzaldehyde, caproaldehyde, carbaldehyde, glutaraldehyde, formaldehyde and the like.

[0057] One skilled in the art will recognize that different cross linking agents may be used at different steps of the present method. Determining acceptable cross linking agents to be used at each step of the present method is well within the abilities of those of skill in the art given the guidance of the teachings herein.

[0058] In an exemplary embodiment, the tissue is treated with substantially any amount of aldehyde that provides the sought after results. The determination of the correct amount of aldehyde needed for a particular application is well within the abilities of those of skill in the art. For example, a biological tissue is contacted one or more times with aldehyde and the biological material is collected and analyzed.

[0059] In an exemplary embodiment, the cross linking agent is formaldehyde. A variety of formaldehyde concentrations are useful in the present invention. In an exemplary embodiment, the tissue is treated with a formaldehyde solution containing from about 0.1% to about 50% formaldehyde by weight. In another exemplary embodiment, the tissue is treated with a formaldehyde solution containing from about 1% to about 10% formaldehyde. In another exemplary embodiment, the tissue is treated with a formaldehyde solution containing about 4% formaldehyde.

[0060] In another exemplary embodiment, the cross linking agent is glutaraldehyde. A variety of glutaraldehyde concentrations are useful in the present invention. In an exemplary embodiment, the tissue is treated with a solution of glutaraldehyde containing from about 0.1% to about 50% of glutaraldehyde. In another exemplary embodiment, the tissue is treated with a solution of glutaraldehyde containing from about 0.2% to about 3% of glutaraldehyde.

[0061] Although certain preferred embodiments of the present invention are illustrated by the use of formaldehyde or glutaraldehyde to mitigate calcification from mammalian tissue, the focus on the use of these two aldehydes is for clarity of illustration and should not be construed as defining or limiting the scope of the invention.

[0062] The skilled practitioner will recognize that other agents are suitable for use in the method of the present invention. An exemplary agent of use in the invention is a cross linking agent. A list of cross linking agents can be found in U.S. Patent No. 6,214,054, which is incorporated herein by reference in its entirety.

Biological Material

[0063] Exemplary biological materials include those at least partially composed of a material of biological origin. Examples of biological materials from a human or an animal include, without limitation, tissue such as brain, muscle (including heart), liver, appendix, pancreas, gastrointestinal tract organs, skin, bone, cartilage, tendon, ligament, connective tissue, and lymphoid tissue such as thymus, spleen, tonsil, lymph nodes, and the like. Alternatively, the biological material may be a biological fluid. The term biological fluid includes, without limitation, to cerebrospinal fluid, blood, serum, plasma, milk, urine, saliva, tears, mucous secretions, sweat, semen and bodily fluids comprising these components. Biological materials may also include culture fluid (or culture medium) used in the production of recombinant proteins or containing cells in suspension prior to transplantation. In another exemplary embodiment, biological materials include products made from human or animal organs or tissues, including serum proteins (such as albumin and immunoglobulin), hormones, food and processed food products, nutritional supplements, bone meal, animal feed, extracellular matrix proteins, gelatin, and other human or animal by products used in manufacturing or final goods. Biological materials may also refer to any material that can be found in a human or animal that is susceptible to infection or that may carry or transmit infection.

[0064] In another exemplary embodiment, the biological material is a bioprosthetic tissue. The bioprosthetic tissue may be incorporated into a bioprosthesis structure for implantation in the body. Bioprosthetic tissue may be comprised entirely of material of biological origin or may additionally comprise material of non-biological origin. One skilled in the art will readily recognize that various methods of assembling a bioprosthesis may be used to incorporate the bioprosthetic tissue into the bioprosthesis. In a further preferred embodiment, the bioprosthetic tissue is a biological tissue.

[0065] Biological tissues may be selected from a variety of tissue types, including substantially any mammalian tissue that is useful in preparing a bioprosthesis having a biological component thereto. For example, in one embodiment, the biological tissue is derived from an organ. In another embodiment, the biological tissue is selected from nerve tissue, glandular tissue (e.g., lymphatic tissue), respiratory tissue, digestive tissue, urinary tract tissue, sensory tissue (e.g., cornea, lens, etc.), and reproductive tissue. In a related embodiment where the biological tissue is a biological fluid, however, addition of liquid is not likely to be necessary, unless to dilute the ionic strength of the biological fluid to permit miscibility of the extraction solvent.

[0066] In an exemplary embodiment, the biological tissue is selected from muscle tissue, adipose tissue, epithelial tissue and endothelial tissue. In another exemplary embodiment, the biological tissue is selected from myocardial tissue and vascular tissue. In a related embodiment, the biological tissue is selected from the group including, without limitation, heart valve, venous valve, blood vessel, aorta, artery, ureter, tendon, dura mater, skin, pericardium, intestine (e.g., intestinal wall),or periosteum. In another exemplary embodiment, the biological tissue is derived from bone, cartilage (e.g. meniscus), tendon, ligament, or any other connective tissue. In another exemplary embodiment, the biological tissue is porcine aortic tissue or bovine pericardium tissue.

[0067] As the source of the biological material used for this purpose may vary with regard to tissue type, the source may also vary with regard to species type (autologous, homologous or heterologous tissue). The artisan will appreciate that the methods of the present invention may be used with bioprosthetic devices that include one or more types of tissues or materials.

[0068] In an exemplary embodiment where the biological material is a solid tissue or product, it may first be suspended in an aqueous solution so that it will be suitable for use in the methods of the present invention. For example, brain tissue may be suspended in sucrose

solution (e.g., 0.32 M sucrose) at 10% weight to volume. Other hypotonic or isotonic solutions include 5% dextrose, phosphate buffered saline, tri-buffered saline, HEPES-buffered saline, or any of the foregoing buffers. The biological material in the aqueous solution can also be homogenized, ground, or otherwise disrupted to maximize contact between the treatment agents and the biological material.

[0069] In another exemplary embodiment, cross linking agents are used to maintain the structural integrity of the biological tissue. Structural integrity can be defined as the ability of tissue to perform its necessary biological function. The artisan will appreciate that the degree of structural integrity required for the tissue to perform its necessary function may vary among different types of tissues. Further, particular applications for which the tissue is used may require different levels of structural integrity.

The SCL Step

[0070] The methods of the present invention include the step of contacting the biological material with a preparation comprising a surfactant and a cross linking agent without denaturant (hereinafter referred to as the SCL preparation). Biological materials, cross linking agents, and surfactants useful in the current invention are discussed above and are likewise useful here in the SCL preparation.

[0071] In an exemplary embodiment more than one SCL step is performed. In another exemplary embodiment, the SCL step is performed from 1 to 5 times. In another exemplary embodiment, the biological material is contacted with the SCL preparation only once.

[0072] A variety of surfactant concentrations are useful in the SCL preparation. In an exemplary embodiment, the tissue is treated with a SCL preparation containing from about 0.1% to about 50% surfactant by weight. In another exemplary embodiment, the tissue is treated with a SCL preparation containing from about 0.1% to about 10% surfactant. In another exemplary embodiment, the tissue is treated with a SCL preparation containing more than 0.6% and less than 50% surfactant. In another exemplary embodiment, the SCL preparation contains from about 1.7% to about 25 % surfactant. In another exemplary embodiment, the SCL preparation contains 0.7%, 0.8%, 0.9%, 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, or 2.9% surfactant. In another exemplary embodiment, the SCL preparation contains 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% surfactant.

[0073] As discussed above, a variety of surfactants are useful in the methods of the present invention, including the SCL preparation. In an exemplary embodiment, the surfactant used in the SCL preparation is a nonionic surfactant. In another exemplary embodiment, the nonionic surfactant is Tween 80. In another exemplary embodiment, the SCL preparation contains 0.7%, 0.8%, 0.9%, 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, or 2.9% of Tween 80. In another exemplary embodiment, the SCL preparation contains 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% of Tween 80.

[0074] One skilled in the art will recognize that different surfactants may be used in the SCL step of the present method. Determining acceptable surfactants and surfactant levels to be used is well within the abilities of those of skill in the art given the guidance of the teachings herein.

[0075] In an exemplary embodiment, the biological tissue is treated with a SCL preparation containing a aldehyde as the cross linking agent. In a related embodiment, the aldehyde is glutaraldehyde. A variety of glutaraldehyde concentrations may be used in the SCL preparation. In an exemplary embodiment, the preparation contains from about 0.1% to about 50% glutaraldehyde by weight. In another exemplary embodiment, the tissue is treated with a SCL preparation containing from about 0.2% to about 3% of glutaraldehyde.

[0076] Typically, the SCL step is performed at any appropriate temperature selected to provide optimal calcification reduction, calcification mitigation, and/or phospholipid content reduction. In exemplary embodiment, the SCL step is performed at approximately a single temperature. In another exemplary embodiment, the SCL step is performed at more than one temperature.

[0077] In another exemplary embodiment, the SCL step is performed at approximately room temperature. In another exemplary embodiment, heat is applied to the SCL preparation. In another exemplary embodiment, the SCL step is performed at a temperature between about 25°C and 55°C. In another exemplary embodiment, the SCL step is performed between about 35°C and 55°C. In another exemplary embodiment, the SCL step is performed between about 35°C and 45°C. In another exemplary embodiment, the SCL step is performed between about 35°C and 40°C. In another exemplary embodiment, the SCL step is performed at a temperature of about 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, or 45°C.

[0078] Typically, the SCL step is performed for a time selected to provide optimal calcification mitigation, calcification reduction or phospholipid content reduction. In an exemplary embodiment, the biological material is contacted with the SCL preparation for about 1 day to about 20 days. In another exemplary embodiment, the SCL step lasts from 5 days to about 15 days.

[0079] In another exemplary embodiment, the SCL step is at least about 12 days. In another exemplary embodiment, the SCL step is at least about 12 days and the SCL preparation comprises at least about 1.7% surfactant.

[0080] In another exemplary embodiment, the SCL step is performed at a pH selected to provide optimal calcification mitigation or phospholipid content reduction. In an exemplary embodiment, the pH of the SCL solution is between about 6 and about 8. In another exemplary embodiment, the pH is maintained at about 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, or 7.9.

[0081] The SCL step can be performed as a single step. Alternatively, the SCL step can be performed as a series of sequential steps. At the end of each sequential step, the SCL preparation is preferably removed from the tissue prior to contacting the tissue with a new fraction of SCL preparation.

The SCLD Step

[0082] The methods of the present invention include the step of contacting the biological material with a preparation comprising a surfactant, a cross linking agent and a denaturant (hereinafter referred to as the SCLD preparation). Biological materials, cross linking agents, and surfactants useful in the current invention are discussed above and are likewise useful here in the SCLD preparation.

[0083] The SCLD step may be performed at different stages in the methods of the current invention. In an exemplary embodiment, the SCLD step is performed after the SCL step. In another exemplary embodiment, the SCLD step is performed before the SCL step.

[0084] The SCLD step may also be referred to as a bioburden reduction step. In an exemplary embodiment, more than one SCLD step is performed. In another exemplary embodiment, two SCLD steps are performed. In an exemplary embodiment where two SCLD steps are performed, the first SCLD step may be referred to as an initial bioburden

reduction step (iBReP) and the second SCLD step may be referred to as a final bioburden reduction step (fBReP).

[0085] A variety of denaturants are useful in the SCLD step of the present invention. In an exemplary embodiment, the denaturant produces a reversible or irreversible loss of higher order structure other than the primary structure of proteins in a biological material. In another exemplary embodiment, the denaturant is a protic solvent useful in removing infectious materials or chemical agents. Protic solvents of use in practicing the present invention include water, alcohols, carboxylic acids, and the like. In another exemplary embodiment, the denaturant is an alcohol or other solvent incorporating an alcohol. The biological material is treated with substantially any amount of alcohol that provides the sought after results.

[0086] The determination of the correct amount of alcohol needed for a particular application is well within the abilities of those of skill in the art. For example, a biological tissue is subjected to one or more SCLD steps and the biological material is collected. The amount of infectious material or chemical agent removed by the SCLD step is determined. When the alcohol ceases to remove infectious agent or chemical agent from the tissue an end point is reached, which is indicative of the amount of alcohol necessary to remove the particular agent from the tissue. In an exemplary embodiment utilizing an alcohol, the tissue is treated with an aqueous alcohol solution containing from about 1% to about 100% alcohol, more preferably from about 10% to about 80% alcohol.

[0087] Exemplary alcohols include one or more compounds of the group consisting of methanol, ethyl alcohol, propyl alcohol, butyl alcohol, pentyl alcohol, hexyl alcohol, heptyl alcohol, octyl alcohol, nonyl alcohol, decyl alcohol, long chain alcohols (e.g. 1,2-octanediol) and the like. In another exemplary embodiment, a method of the present invention uses ethanol. In another exemplary embodiment, the alcohol is isopropyl alcohol.

[0088] As discussed above, a variety of cross linking agents are useful in the methods of the present invention, including the SCLD preparation. In an exemplary embodiment, the SCLD preparation comprises an aldehyde as the cross linking agent. In a further exemplary embodiment, formaldehyde is the cross linking agent. A variety of formaldehyde concentrations are useful in the SCLD preparation. In an exemplary embodiment, the SCLD preparation comprises about 0.5% to about 10% formaldehyde. In another exemplary embodiment, the SCLD preparation comprises about 4% formaldehyde.

[0089] The SCLD preparation may employ any appropriate surfactant. In an exemplary embodiment, the surfactant used in the SCLD preparation is a nonionic surfactant. In another exemplary embodiment, the SCLD preparation comprises Tween 80 as the surfactant. In another exemplary embodiment, the SCLD preparation comprises about 0.5% to about 5% Tween 80. In another exemplary embodiment, the SCLD preparation comprises about 2.1% of Tween 80.

[0090] In an exemplary embodiment, the SCLD step is performed at a temperature selected to provide optimal calcification mitigation, calcification reduction and/or reduction of phospholipid content of a biological material. In exemplary embodiment, the SCLD step is performed at approximately a single temperature. In another exemplary embodiment, the SCLD step is performed at more than one temperature.

[0091] In another exemplary embodiment, the SCLD step is performed at approximately room temperature. In another exemplary embodiment, heat is applied to the SCLD preparation. In another exemplary embodiment, the SCLD step is performed at a temperature between about 30°C and 55°C. In another exemplary embodiment, the SCLD step is performed between about 30°C and about 40°C.

[0092] In another exemplary embodiment, two SCLD steps are preformed: an iBReP step and an fBReP step. In another exemplary embodiment, the iBReP step is performed at about room temperature and the fBReP step is performed at a temperature above room temperature. In another exemplary embodiment, the fBReP step is performed between about 30°C and about 37°C.

[0093] In another exemplary embodiment, each SCLD step is performed for a time selected to provide optimal calcification mitigation, calcification reduction and/or reduction of phospholipid content of a biological material. In another exemplary embodiment, the iBReP step is performed from about 1 hour to about 4 hours and the fBReP step is performed from about 5 hours to about 15 hours.

[0094] In another exemplary embodiment, the SCLD step is performed at a pH selected to provide optimal calcification mitigation, calcification reduction and/or reduction of phospholipid content of a biological material. In another exemplary embodiment, the pH of the SCLD solution is between about 6 and about 8. In another exemplary embodiment, the pH is maintained at about 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, or 7.9.

[0095] The SCLD step can be performed as a single step. Alternatively, the extraction can be performed as a series of sequential steps. At the end of each sequential step, the SCLD preparation is preferably removed prior to contacting the tissue with a new fraction of the SCLD preparation.

[0096] In an exemplary embodiment, the SCLD step is performed before the SCL step. In another exemplary embodiment, the SCLD step is performed after the SCL step.

Contacting a Biological Material with a Terminal Liquid Sterilization Solution

[0097] In an exemplary embodiment, the methods of the present invention further include contacting the biological tissue with a terminal liquid sterilization solution. Typically, the biological tissue is contacted with a terminal liquid sterilization solution after completion of the SCL and SCLD steps previously discussed.

[0098] Exemplary terminal liquid sterilization solutions (hereinafter referred to as "TLS" solutions) are capable of sterilizing the biological material before implantation. Sterilants useful in the current invention include cross linking agents, heat, radiation, electron beams, ultraviolet radiation, and combinations thereof. In an exemplary embodiment, the TLS solution comprises heat and a cross linking reagent. Exemplary cross linking agents useful in the TLS solution are described above.

[0099] In an exemplary embodiment, the TLS solution is heated to a temperature from about 35°C to about 50°C. In another exemplary embodiment, TLS solution is heated to a temperature from about 35°C to about 40°C.

[0100] In another exemplary embodiment, the TLS solution is maintained at a pH between about 6 and 9. In another exemplary embodiment, the TLS solution is maintained at a pH between about 7.2-7.5.

[0101] In an exemplary embodiment, the TLS solution is contacted with the biological material for sufficient time to sterilize the biological material. One of skill will recognize that the time required for sterilization will depend on the stringency of the TLS solution. For example, a TLS solution containing an aldehyde that is heated to about 37°C may require 1 to 6 days to sterilize the biological material whereas heating the TLS solution to 50°C may decrease the required time to 25 hours.

[0102] In another exemplary embodiment, the TLS solution comprises about 0.2% to about 2.0% by weight glutaraldehyde.

[0103] In another exemplary embodiment, the terminal sterilization time and temperature is 1-6 days at 37 degrees Celsius. In another particularly preferred embodiment, the terminal sterilization time and temperature is 1-2 days at 50 degrees Celsius. One skilled in the art will recognize that other terminal sterilization times and temperatures may be adjusted while maintaining the effectiveness of the TLS solution.

Contacting a Biological Material with a Cross Linking Agent

[0104] In an exemplary embodiment, the methods of the present invention further include contacting the biological tissue with a fixation solution that comprises a cross linking agent in the absence of denaturant. The biological tissue is contacted with the fixation solution before the SCL and SCLD steps, after the SCL and SCLD steps, or between the SCL and SCLD steps. Typically, the biological tissue is contacted with the fixation solution before the SCL and SCLD steps.

[0105] Fixation solutions useful in the present invention include a cross linking agent to effect crosslinking of the connective tissue proteins within the tissue. Cross linking agents useful in the current invention are discussed above and are likewise useful here in the fixation solution.

[0106] In an exemplary embodiment, the fixation solution contains a cross linking agent at a concentration of about 0.2% to about 2.0% by weight. In another exemplary embodiment, the cross linking reagent is glutaraldehyde. In another exemplary embodiment, the fixation solution contains glutaraldehyde as the cross linking agent at a concentration of about 0.2% to about 2.0% by weight.

[0107] In another exemplary embodiment, the fixation solution is contacted with the biological material from about 0.5 hours to about 14 days. In another exemplary embodiment, the fixation solution is contacted with the biological material for about 1-4 days. In another exemplary embodiment, the fixation solution is contacted with the biological material for about 2 days.

[0108] In another exemplary embodiment, the fixation solution further comprises a surfactant. Useful surfactants and surfactant concentrations are described above and are equally applicable to the fixation solution.

[0109] The terms and expressions which have been employed herein are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding equivalents of the features shown and described, or portions thereof, it being recognized that various modifications are possible within the scope of the invention claimed. Moreover, any one or more features of any embodiment of the invention may be combined with any one or more other features of any other embodiment of the invention, without departing from the scope of the invention. For example, the surfactants and cross linking agents and respective concentrations described in the SCL step are equally applicable to the SCLD step. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

EXAMPLE 1

[0110] Example 1 describes a method of mitigating calcification in pericardial tissue wherein the pericardial tissue is subjected to treatment with a SCL preparation comprising 10% Tween 80 and 0.625% glutaraldehyde.

1.1. Methods

1.1.1. Post Tween Process Method

[0111] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0112] After preparation of the bovine pericardial tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 30 minutes followed by a 7 day quarantine.

[0113] Remaining leaflets were treated with 0.625% glutaraldehyde and 10% Tween 80 in aqueous solvent for 7 days. Leaflets were then either subjected to subsequent steps or implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis. The leaflets that were implanted into rats are hereinafter referred to in Example 1 as "Post Tween Process" leaflets.

[0114] Remaining leaflets were subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 to 2.5 hours.

[0115] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0116] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2%

Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 to 37 degrees Celsius for 10 hours ± 30 minutes.

[0117] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0118] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35-40°C (measured at representative coolest and warmest product internal location) for 25 – 35C.

[0119] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 1 as "Tween/TLS Process" leaflets.

1.1.2. Full Process Control Method

[0120] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0121] After preparation, the leaflets were chemically fixed by treatment with 0.625% glutaraldehyde in aqueous solvent for 14 days. Leaflets were then either subjected to subsequent steps or implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis. The leaflets that were implanted into rats are hereinafter referred to in Example 1 as "14-day Control" leaflets.

[0122] Remaining leaflets were subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0123] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0124] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0125] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0126] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35 - 40°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours including.

[0127] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 1 as "Full Process Control" leaflets.

1.1.3. Calcium Analysis

[0128] After undergoing one of the four treatments outlined above, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days. The leaflets were removed and prepared for calcium content analysis.

[0129] Leaflets were first lyophilized and weighed then treated with 70% Nitric Acid at 110°C until completely digested. The digested samples were brought to a final concentration of 3,000 ppm of KCl.

[0130] The samples were analyzed for calcium content by atomic absorption spectroscopy (AAS).

1.2. Results

[0131] Figure 1 shows the total μg Ca/mg dry leaflet tissue for repetitive treatments using the four methods described above: 1) 14-day Control; 2) Full Process Control; 3) Post Tween Process; and 4) Tween/TLS Process. The data in Figure 1 shows a reduction in the average calcium content of the Tween/TLS Process and the Post Tween Process relative to the Full Process Control and 14-day Control.

[0132] Figure 2 shows a diamond plot representation of the average calcium content decrease data in Figure 1. The top and bottom of the diamonds in Figure 2 form the 95% confidence interval for the means. The small squares are individual data points. The small horizontal lines near the top and bottom of each diamond are overlap lines.

[0133] Table 1 shows the mitigation of calcification using the Post Tween/TLS step relative to the Full Process Control Leaflet tissue samples containing more than 5 μg Ca/mg dry leaflet tissue are calcified.

Table 1

Method	No Calcification	Calcification	Total Samples
Full Process Control	59	5	64
Post Tween/TLS	62	0	62

[0134] Table 1 shows that 7.8% of the tissue from the Full Process Control (group 2) calcified while 0% from the Tween/TLS Process group (group 4) calcified.

EXAMPLE 2

[0135] Example 2 describes a method of mitigating calcification in porcine heart valve tissue wherein the porcine heart valve tissue is subjected to treatment with a SCL preparation comprising 10% Tween 80 and 0.625% glutaraldehyde.

2.1. Methods

2.1.1. Post Tween Process Method

[0136] Porcine heart valve tissue leaflets were prepared for chemical fixation by standard techniques.

[0137] After preparation of the porcine heart valve tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 7 days.

[0138] Remaining leaflets were treated with 0.625% glutaraldehyde and 10% Tween 80 in aqueous solvent for 7 days. Leaflets were then either subjected to subsequent steps or implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis. The leaflets that were implanted into rats are hereinafter referred to in Example 2 as "Post Tween Process" leaflets.

[0139] Remaining leaflets were then subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0140] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0141] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0142] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0143] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35 - 40°C (measured

at representative coolest and warmest product internal location) for 25 – 35 hours including heat up and cool down within 35 - 40°C.

[0144] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 2 as "Tween/TLS Process" leaflets.

2.1.2. Full Process Control Method

[0145] Porcine heart valve leaf tissue were prepared for chemical fixation by standard techniques.

[0146] After preparation, the leaflets were chemically fixed by treatment with 0.625% glutaraldehyde in aqueous solvent for 14 days. Leaflets were then either subjected to subsequent steps or implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis. The leaflets that were implanted into rats are hereinafter referred to in Example 2 as "14-day Control" leaflets.

[0147] Remaining leaflets were subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0148] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0149] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0150] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or

greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0151] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 37.5 ± 2.5°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours.

[0152] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 2 as "Full Process Control" leaflets.

2.1.3. Calcium Analysis

[0153] After undergoing one of the four treatments outlined above, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days. The leaflets were removed and prepared for calcium content analysis.

[0154] Leaflets were first lyophilized and weighed then treated with 70% Nitric Acid at 110°C until completely digested. The digested samples were brought to a final concentration of 3,000 ppm of KCl.

[0155] The samples were analyzed for calcium content by atomic absorption spectroscopy (AAS).

2.2. Results

[0156] Figure 3 shows the total μg Ca/mg dry leaflet tissue for repetitive treatments using the four methods described above: 1) 14-day Control; 2) Full Process Control; 3) Post Tween Process; and 4) Tween/TLS Process. The data in Figure 3 shows a reduction in the average calcium content of the Tween/TLS Process relative to the Full Process Control and 14-day Control.

[0157] Figure 4 shows a diamond plot representation of the average calcium content decrease data in Figure 3. The top and bottom of the diamonds in Figure 2 form the 95% confidence interval for the means. The small squares are individual data points. The small horizontal lines near the top and bottom of each diamond are overlap lines.

[0158] Table 2 shows the mitigation of calcification using the Post Tween/TLS step relative to the Full Process Control. Leaflet tissue samples containing more than 5 μg Ca/mg dry leaflet tissue are calcified.

Table 2

Method	No Calcification	Calcification	Total Samples
Full Process Control	28	7	35
Post Glut/Tween Full Process Test	34	3	37

[0159] Table 2 shows that 20% (7/35) of the tissue from the Full Process Control (group 2) calcified while 8.1% (3/37) from the Tween/TLS Process group (group 4) calcified.

EXAMPLE 3

[0160] Example 3 describes a method of mitigating calcification in pericardial tissue wherein the pericardial tissue is subjected to treatment with a SCL preparation comprising 1.2% Tween 80 and 0.625% glutaraldehyde.

3.1. Methods

3.1.1. Control Group, 14 Day Glutaraldehyde Treated Leaflets

[0161] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0162] After preparation of the bovine pericardial tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 12-24 hours followed by a 14 day quarantine. The samples were then implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis. These samples are hereinafter referred to in Example 3 as "Group 1" leaflets.

3.1.2. Control Group, Fully Processed Leaflets

[0163] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0164] After preparation of the bovine pericardial tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 12-24 hours followed by a 14 day quarantine.

[0165] The leaflets were then subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0166] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0167] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0168] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0169] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 37.5 ± 2.5°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours.

[0170] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred in Example 3 as "Group 2" leaflets.

3.1.3. Test Group, 14 Day Glutaraldehyde, 7 Day Glutaraldehyde/1.2% Tween-80 Treated Leaflets

[0171] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0172] After preparation of the bovine pericardial tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 12-24 hours followed by a 7 day quarantine. Subsequently, the leaflets were treated with 0.625% glutaraldehyde and 1.2% Tween 80 in aqueous solvent for 7 days.

[0173] The leaflets were then subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0174] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0175] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4 % Formaldehyde, 22 % ethanol, 1.2 % Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0176] After treatment, the leaflets were rinsed in 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0177] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the

solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35 - 40°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours.

[0178] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 3 as "Group 3" leaflets.

3.1.4. Test Group, 1 Day Glutaraldehyde, 13 Day Glutaraldehyde/1.2% Tween-80 Treated Leaflets

[0179] Bovine pericardial tissue leaflets were prepared for chemical fixation using standard techniques.

[0180] After preparation of the bovine pericardial tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 12-24 hours. Subsequently, the leaflets were treated with 0.625% glutaraldehyde and 1.2% Tween 80 in aqueous solvent for 13 days.

[0181] The leaflets were then subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80, and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0182] After treatment, the leaflets were rinsed in 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0183] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4 % Formaldehyde, 22 % ethanol, 1.2 % Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0184] After treatment, the leaflets were rinsed in 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or

greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0185] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.625 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35 40°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours.

[0186] After the TLS procedure, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days, explanted, and submitted for calcium analysis and are hereinafter referred to in Example 3 as "Group 4" leaflets.

3.1.3. Calcium Analysis

[0187] After undergoing one of the four treatments outlined above, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days. The leaflets were removed and prepared for calcium content analysis.

[0188] Leaflets were first lyophilized and weighed then treated with 70% Nitric Acid at 110°C until completely digested. The digested samples were brought to a final concentration of 3,000 ppm of KCl.

[0189] The samples were analyzed for calcium content by atomic absorption spectroscopy (AAS).

3.2. Results

[0190] Table 3 shows the mitigation of calcification using the 7 day Tween 80 test group (group 3) and 13 day Tween 80 test group (group 4) relative to the Full Process Control (group 2). Leaflet tissue samples containing more than 5 μg Ca/mg dry leaflet tissue are calcified.

Table 3

Method	No Calcification	Calcification	Total Samples
Group 2	90	4	94
Group 3	91	0	91
Group 4	87	2	89

[0191] Table 2 shows that 4.26% (4/94) of the tissue from the Full Process Control (group 2) calcified while 0.0% (0/91) from the 7 day Glutaraldehyde, 7 day Tween 80 test group (group 3) calcified. In addition, only 2.25% of the 1 day Glutaraldehyde, 13 day Tween 80 test group (group 4) showed calcification. Thus, both group 3 and 4 showed a reduction in calcification relative to the Full Process Control groups.

EXAMPLE 4

[0192] Example 4 describes a method of reducing phospholipid content in porcine heart valve tissue wherein the porcine heart valve tissue is subjected to treatment with a SCL preparation comprising between 0.6%-6% Tween 80 and 0.625% glutaraldehyde for 7 to 15 days.

4.1. Methods

4.1.1. General Methods

[0193] Porcine heart valve tissue leaflets were prepared for chemical fixation by standard techniques.

[0194] After preparation of the porcine heart valve tissue leaflets, the samples were chemically fixed by immersion into a solution of 0.625% glutaraldehyde in aqueous solvent for 1 to 14 days as shown in Table 4.

[0195] Leaflets were then treated with 0.625% glutaraldehyde and from 0.6% to 6.0% Tween 80 for 5 to 15 days as shown in Table 4.

[0196] Leaflets were then subjected to an initial bioburden reduction process (iBReP). In the iBReP procedure, leaflets were treated with a solution of formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at room temperature for 2 - 2.5 hours.

[0197] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 3 consecutive cycles of 10 minutes each.

[0198] After the iBReP procedure, leaflets were subjected to a final bioburden reduction process (fBReP). In the fBReP procedure, leaflets were treated with a solution of

formaldehyde-based sterilant. The solution contained 4% Formaldehyde, 22% ethanol, 1.2% Tween 80 and 11.3 mM phosphate buffer. The volume was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Leaflets were treated at 30 - 37 degrees Celsius for 10 hours ± 30 minutes.

[0199] After treatment, the leaflets were rinsed in 0.625% glutaraldehyde in phosphate buffer in aqueous solvent at 7.4 pH. The volume of the rinsing solution was equal to or greater than one hundred milliliters (100 ml) of solution per 3 leaflets. Rinsing was performed at room temperature for 4 consecutive cycles of 10 minutes each.

[0200] After the fBReP procedure, leaflets were subjected to a terminal liquid sterilization process (TLS). In the TLS procedure, leaflets were treated with a solution of 0.516 - 0.656 % glutaraldehyde in phosphate buffer in aqueous solvent at 7.2-7.5 pH. The volume of the solution was 100 ml of solution per 3 leaflets. Leaflets were treated at 35 - 40°C (measured at representative coolest and warmest product internal location) for 25 – 35 hours.

4.1.2. Phospholipid Analysis

[0201] After undergoing the treatment outlined above, leaflets were implanted subcutaneously into Sprague-Dawley Rats for 90 days. The leaflets were explanted and prepared for phospholipid content analysis.

[0202] Leaflets were frozen liquid in nitrogen, pulverized, and dissolved in extraction solvent containing chloroform and methanol. After addition of physiological saline, the precipitated proteins and bulk tissue were removed by centrifugation. The extracted lipids were then collected from the solvent phase and subjected to thin layer chromatography analysis.

[0203] The selected phospholipids analyzed by TLC were sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid.

4.2. Results

[0204] Table 4 shows the reduction of phospholipid content in methods using the SCL step relative to the method where no SCL step is performed.

Table 4

Fixation conditions	SCL conditions	phospholipid content
0.625% Glutaraldehyde; 7 days	1.7% Tween; 7days; 25°C	++
0.625% Glutaraldehyde; 7 days	1.7% Tween; 7days; 37°C	+++
0.625% Glutaraldehyde; 7 days	4.9% Tween; 7days; 25°C	+++
0.625% Glutaraldehyde; 7 days	4.9% Tween; 7days; 37°C	+++
0.625% Glutaraldehyde; 1 day	1.7% Tween; 13 days; 25°C	+++
0.625% Glutaraldehyde; 1 day	1.7% Tween; 13 days; 37°C	+++
0.625% Glutaraldehyde; 1 day	4.9% Tween; 13 days; 25°C	+++
0.625% Glutaraldehyde; 1 day	4.9% Tween; 13 days; 37°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 31°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 31°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 31°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 31°C	+++
0.625% Glutaraldehyde; 9 days	3.3% Tween; 5 days; 31°C	+++
0.625% Glutaraldehyde; 1 day	3.3% Tween; 15 days; 31°C	+++
0.625% Glutaraldehyde; 4 days	0.6% Tween; 10 days; 31°C	++

0.625% Glutaraldehyde; 4 days	6.0% Tween; 10 days; 31°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 25°C	+++
0.625% Glutaraldehyde; 4 days	3.3% Tween; 10 days; 37°C	+++
0.625% Glutaraldehyde; 14 days	No SCL Treatment	---
0.625% Glutaraldehyde; 7 days	1.2% Tween; 7 days; 25°C	+++

+++ represents <0.200 μg PL/mg of dried tissue

++ represents >0.200 μg PL/mg of dried tissue and <0.400 μg PL/mg of dried tissue

+ represents > 0.400 μg PL/mg of dried tissue and <1.000 μg PL/mg of dried tissue

--- represents >1.00 μg PL/mg of dried tissue